

S17834, a New Inhibitor of Cell Adhesion and Atherosclerosis That Targets NADPH Oxidase

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Abstract—Oxidant stress is involved in the events that accompany endothelial cell expression of adhesion molecules and leukocyte adherence in many disease states, including atherosclerosis. A recently discovered benzo(b)pyran-4-one derivative, S17834 (10 to 50 $\mu\text{mol/L}$), reduced tumor necrosis factor-stimulated vascular cell adhesion molecule-1 (VCAM) mRNA accumulation and protein expression in human umbilical vein endothelial cells. Intercellular cell adhesion molecule-1 and E-selectin were also inhibited by S17834, but platelet endothelial cell adhesion molecule-1 was not. Adherence of U937 monocytic cells to the endothelial cells as well as to plastic plates coated with soluble VCAM, intercellular cell adhesion molecule-1, P-selectin, and E-selectin was also decreased. Consistent with an antioxidant mechanism of action, S17834 (10 to 50 $\mu\text{mol/L}$) inhibited tumor necrosis factor-stimulated release of superoxide from endothelial cells measured by cytochrome *c* reduction. S17834 had no effect on superoxide produced by xanthine oxidase, indicating that rather than by acting as a scavenger of superoxide anion, the drug acts by inhibiting the production of free radicals. Indeed, S17834 inhibited NADPH oxidase activity of endothelial cell membranes. The ability to inhibit superoxide anion production appears to be key in the effect of S17834 on superoxide anion production and VCAM expression, because these actions were mimicked by adenovirus-mediated overexpression of superoxide dismutase. Furthermore, these actions may be relevant in vivo, because S17834 reduced aortic superoxide anion levels by 40% and aortic atherosclerotic lesions by 60% in apolipoprotein E-deficient mice. These results indicate that S17834 inhibits adhesion molecule expression and adherence of leukocytes to endothelial cells as well as aortic atherogenesis and that perhaps these effects can be explained by its ability to inhibit endogenous superoxide anion production. (*Arterioscler Thromb Vasc Biol.* 2001;21:1577-1584.)

Key Words: NADPH oxidase ■ superoxide anion ■ adhesion molecules ■ atherosclerosis

The inflammatory response consists of a complex sequence of events resulting in leukocyte extravasation from the circulation into tissues.¹ In the microcirculation, leukocyte-endothelial cell interactions at the level of post-capillary venules represent a pivotal step in this process.² A similar process in large arteries has been implicated in the infiltration of monocytes, followed by accumulation of lipids and development of atherosclerotic plaques.³ The influx of leukocytes into the vasculature involves several endothelial and leukocyte adhesion receptors with specific functions. Some of these are dependent on cell activation by local tissue and circulating cytokines, whereas others are constitutively expressed. Intracellular oxidative stress is thought to regulate leukocyte adhesion to postcapillary venules after ischemia/reperfusion,⁴⁻⁶ and recent evidence points to the involvement of oxygen-derived free radicals in the expression of endothelial cell adhesion molecules (CAMs),⁷ including expression in the context of atherosclerosis.⁸

Because leukocyte-endothelial cell interactions may participate in the initiation or the progression of several chronic diseases, including atherosclerosis, diabetes, and venous insufficiency, the mediators involved become targets for new drugs. Recent studies have shown that 6,8-diallyl 5,7-dihydroxy 2-(2-allyl 3-hydroxy 4-methoxyphenyl)-1-H benzo(b)pyran-4-one (S17834) inhibited ischemia/reperfusion-induced leakage of macromolecules and leukocyte adhesion in postcapillary venules of the hamster cheek pouch.^{9,10} In the present study, we investigated the mechanism by which S17834 inhibits the stimulation by tumor necrosis factor- α (TNF) of mRNA and protein expression of vascular CAM-1 (VCAM), intercellular CAM-1 (ICAM), and E-selectin in human umbilical vein endothelial cells (HUVECs), as well as leukocyte adherence mechanisms. We found that S17834 decreased endogenous superoxide anion production in TNF-stimulated endothelial cells. Consistent with a similar in vivo effect of the drug, apoE-deficient mice

Received May 29, 2001; revision accepted July 9, 2001.

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treated with S17834 demonstrated decreased superoxide anion and atherosclerotic lesions in the aorta. These studies suggest that S17834 inhibits cytokine-stimulated expression of CAMs and the adherence of leukocytes to endothelium and, by consequence of these effects *in vivo*, inhibits atherogenesis.

Methods

Materials, Reagents, Antibodies, and Antigens

Diethylthiocarbamate, superoxide dismutase (SOD) from bovine erythrocytes, 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron), EDTA, and phorbol 12-myristate 13-acetate were obtained from Sigma Chemical Co. Anhydrous dimethyl sulfoxide (DMSO) was obtained from Aldrich Chemical Co. S17834 was obtained from the Institut de Recherches Servier.

TNF (specific activity 2×10^7 U/mg) and primary monoclonal mouse anti-human VCAM and ICAM antibody (IgG1) were purchased from Genzyme. Human recombinant soluble P-selectin (sP-selectin), soluble E-selectin (sE-selectin), soluble VCAM (sVCAM), and soluble ICAM (sICAM) were all obtained from R&D Systems.

All reagents were prepared fresh and kept on iced until needed.

Human Endothelial and Monocyte Cell Culture

HUVECs and human arterial endothelial cells were obtained from Clonetics Co as cryopreserved cell suspensions. Between the third and sixth passages, endothelial cells were used to seed 96-well Costar plates at a density of ≈ 5000 cells per well for VCAM protein analysis or 162-cm² culture flasks at a density of ≈ 5000 cells/cm² for CAM mRNA expression. Monolayers were used at confluence within 5 days for all experiments.

U937 monocytic cells (CRL 1593.2, American Type Culture Collection) were grown in RPMI 1640 medium (Sigma) supplemented with 10% FCS (GIBCO), 1% penicillin-streptomycin (GIBCO), sodium pyruvate (1 mmol/L), and 10 mmol/L HEPES (GIBCO) at 37°C in a 5% CO₂ humidified atmosphere. HSB₂ leukocytes (American Type Culture Collection) were grown in suspension in DMEM modified by Iscove (GIBCO) supplemented with 10% FCS (Dutsher), 1% penicillin-streptomycin (GIBCO), 1% L-glutamine (GIBCO), and 12.5 mmol/L HEPES at 37°C in a 5% CO₂ humidified atmosphere. For both cell types, culture media were replaced every 2 days. For the adhesion assays with recombinant CAM, cells were thawed from frozen stocks and thus were in the same growth state in each experiment. Cell suspensions were separated into aliquots, frozen, and kept for a maximal period of 2 months at -80°C before experimentation.

Endothelial Cell Treatments

For VCAM expression, endothelial cells in phenol-free endothelial basal medium (Clonetics) with 0.2% human serum albumin were stimulated with TNF (100 U/mL) for 6 hours at 37°C in a humidified 5% CO₂ atmosphere. As specified in the text, other parameters were measured from 2 to 6 hours after TNF when the parameter being measured was found to be maximal. S17834 was solubilized in a stock solution in either NaOH with an equal volume of HCl added after solubilization or in 100% DMSO and then diluted 1:1000 in endothelial basal medium (Clonetics Co) so that the final concentration of DMSO was $<0.1\%$. Solvent controls were routinely performed in which the cells were exposed to the highest concentration of solvent used. Cytotoxicity was evaluated by lactate dehydrogenase activity released by cells in culture medium (50 μL) collected at the end of the incubation period (Cytotox 96 nonradioactive cytotoxicity assay, Promega). In the experiments reported, S17834 did not significantly increase the lactate dehydrogenase activity found in the supernatant compared with the solvent alone, and no detachment of cells from the plates was observed.

VCAM Cell Surface Expression

The expression of VCAM on the surface of endothelial cells was analyzed with an Ascent fluorometer plate reader (Laboratory

Systems Corp) by use of a fluorescent ELISA to allow for the detection of VCAM directly on the cell surface as previously described.¹¹ VCAM expression was calculated in arbitrary units of fluorescence. Measurements of VCAM surface expression were repeated on at least 3 occasions, each time in triplicate.

Total RNA Extraction and Northern Blot Analysis

Total RNA was extracted from cells by use of the total RNA isolation system kit (Promega). For Northern blot analysis, 20 μg total RNA was separated on a 1% denaturing formaldehyde agarose gel and then transferred to a nylon membrane (Pall). Prehybridization and hybridization with ³²P-labeled probes were performed at 42°C in 6-fold concentrated saline-sodium citrate (0.3 mol/L sodium citrate and 3 mol/L NaCl, pH 7.0), 5 \times concentrated Denhardt's solution (0.1% BSA, 0.1% Ficoll, and 0.1% polyvinylpyrrolidone), 0.1% SDS, and 0.1 mg/mL boiled salmon sperm DNA. The oligonucleotide probes encoding human ICAM, human VCAM, human E-selectin, and human platelet endothelial CAM (PECAM) were obtained from R&D systems. Transcript signals were quantified by densitometry with gel-analysis software (Imager soft 1D&2D, Appligene). The density of mRNA bands was expressed as relative area and was normalized by dividing the area of an individual mRNA band by that of the β -actin mRNA band.

Endogenous Endothelial Cell Superoxide Anion Generation

Superoxide anion released by intact endothelial cell monolayers in phenol-free culture medium was determined by measuring the SOD-inhibitable reduction of acetylated horse heart type VI cytochrome C (80 $\mu\text{mol/L}$) to the ferrous form at a wavelength of 550 nm. Reduction of cytochrome c was carried out at pH 7.4 in the presence of the iron chelator EDTA (100 $\mu\text{mol/L}$) and catalase (1000 U/mL) to eliminate any potential metal ion and hydrogen peroxide-dependent effect. SOD was used at a concentration of 2500 U/mL. In addition, a sample of culture medium that had been incubated without cells under the same conditions was used as a blank. The superoxide anion production was calculated by converting the difference in optical density between samples with and without SOD and using the molar extinction coefficient for cytochrome c at 550 nm (29.5 [mmol/L]⁻¹ · s⁻¹). Superoxide anion production was expressed as nanomoles per minute per milligram of protein.

Monocyte Adherence to Endothelial Cells

An optimal concentration of U937 cells in suspension ($5 \times 10^6/\text{mL}$) was labeled with Calcein (20 $\mu\text{mol/L}$, Molecular Probes) before the adhesion assay by incubation for 30 minutes at 37°C. Labeled U937 cells were added to individual wells for adherence assays as previously described.¹¹ Adherence was quantified as the number of adherent monocytes per well.

Adenovirus-Mediated Overexpression of CuZnSOD and Catalase

Replication-defective recombinant adenovirus was obtained from the Vector Core Laboratory at the University of Iowa. Adenoviral vectors carrying the cDNA for human copper/zinc-SOD (Ad-CuZnSOD) and human catalase (Ad-Cat) and control virus encoding the *Escherichia coli* LacZ gene (Ad-LacZ) were incubated with endothelial cells at a multiplicity of infection (MOI) of 300. The transfection protocol included an initial 2-hour infection in serum-free endothelial cell culture medium supplemented with 0.2% human serum albumin, followed by 22 hours with medium containing 2% FBS. At 24 hours, the medium was changed, and at 48 hours, the cells were stimulated with TNF. This protocol allowed for maximal increase in SOD and catalase activity while allowing for basal expression of VCAM to return to baseline after an initial rise that was observed during the first 24 hours.

SOD and Catalase Activity

Initial experiments with adenoviral vectors determined that maximum enzyme activity was reached 48 hours after transfection.

Endothelial cell lysate was obtained after sonication with 6 bursts of 10 seconds at 20 W under ice in 10 mmol/L Tris-Cl, pH 8.2, containing 0.34 mol/L sucrose, 1 mmol/L diethylenetriamine pentaacetic acid, 10 $\mu\text{g}/\text{mL}$ pepstatin, 10 $\mu\text{g}/\text{mL}$ aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, and 10 $\mu\text{g}/\text{mL}$ leupeptin. SOD activity was determined by the inhibition of O_2^- -dependent auto-oxidation of pyrogallol,¹² carried out at pH 8.2 in 50 mmol/L Tris-HCl buffer at room temperature. The chelating agent, diethylenetriamine pentaacetic acid, was added to the assay buffer to lessen auto-oxidation catalyzed by metal ions, and 1200 U/mL catalase was added to prevent inactivation of SOD by H_2O_2 formed in the reaction. Pyrogallol autooxidation under these conditions is a linear reaction and leads to the formation of a colored product that can be followed at an absorbance peak at 420 nm. Total SOD activity in cell lysate was calculated against a standard curve by using a range of SOD (bovine erythrocytes) concentrations (0 to 2 U) and was expressed as units per milligram cell protein. Catalase activity was determined by measuring the rate of decrease in absorbance of 1% hydrogen peroxide at 240 nm in sodium phosphate buffer (50 mmol/L, pH 7) with EDTA (100 $\mu\text{mol}/\text{L}$). Aliquots of cell lysate were compared with catalase standards (0.1 to 1 U/mL). Protein was determined after centrifugation of the cell lysate for 5 minutes at 800g by use of the Bradford assay.

Adhesion Assay on Recombinant CAM

Wells of microplates were coated overnight at 4°C with 5 $\mu\text{g}/\text{mL}$ of human recombinant sP-selectin, sE-selectin, sVCAM, or sICAM in a PBS solution. After washings with PBS, free sites on the polystyrene wells were saturated with PBS containing 1% BSA (PBS-BSA). In the case of wells coated with sP-selectin, sE-selectin, or sVCAM, S17834 or its vehicle (diluted in PBS-BSA) was added in the presence of 10^6 U937 human monocytic cells/mL (CRL 1593.2, American Type Culture Collection) in PBS-BSA for 90 minutes at 20°C. In the case of wells coated with sICAM-1, S17834 or its vehicle was added to 10^6 HSB₂ human lymphocytic cells/mL (CCL 120.1, American Type Culture Collection) in the presence of 100 ng/mL phorbol 12-myristate 13-acetate in PBS-BSA for 90 minutes at 20°C. The supernatant was then removed and kept to determine the direct toxicity of the compound on the cells. The wells were then washed carefully 3 times with PBS. Adhesive cells were then lysed, and the lactate dehydrogenase activity contained in each well was determined by using the Cytotox 96 kit (Promega). The adhesive cells were then quantified versus a calibration curve performed with wells containing increasing known concentrations of lysed U937 or HSB₂ cells.

The estimation of the concentration inhibiting 50% of adherent cells (IC_{50}) was performed using nonlinear regression: $E = (E_{\text{max}} \times C^{\text{nh}}) / (\text{IC}_{50}^{\text{nh}} + C^{\text{nh}})$, where E is the effect; E_{max} , the maximal effect; C, the concentration of S17834; and nh, the Hill coefficient.

NADPH Oxidase Activity

HUVECs were treated with TNF (100 U/mL) for 4 hours, and diethyldithiocarbamate (10 mmol/L) was added for the last 15 minutes. Cells were washed 3 times with HBSS, harvested, and resuspended in 10 mmol/L Tris plus 0.3 mol/L sucrose buffer (pH 7.1). The suspension was later sonicated (3 times for 10 seconds each) on ice and centrifuged at 15 000g for 10 minutes. The supernatant was centrifuged again at 100 000g for 60 minutes. The pellet was resuspended in Tris-sucrose buffer. Superoxide production was measured in 100 mmol/L phosphate buffer containing 100 μg particulate fraction, 100 $\mu\text{mol}/\text{L}$ NADPH, 80 $\mu\text{mol}/\text{L}$ acetylated cytochrome c, 1000 U/mL catalase, and 100 $\mu\text{mol}/\text{L}$ EDTA. The change in absorbance at 550 nm was recorded over 30 minutes. After subtracting the rate of absorbance of samples containing 1000 U/mL SOD, results were expressed as nanomoles per minute per milligram by using the extinction coefficient for cytochrome c.

Animal Protocol and Diet

Female homozygous apoE-deficient mice (backcrossed for at least 10 generations to the C57BL/6J background) were obtained at 8 weeks of age from Jackson Laboratories (Bar Harbor, Me). The mice were fed normal mouse chow (Certified Rodent Chow 5002, Purina

containing 4.5% fat and given free access to food and water throughout the study. After 1 week of acclimatization, some mice were given S17834 (130 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), which was mixed with crushed normal mouse chow and pelleted again. The dose of drug was calculated on the basis of the average consumption of food (5 g/d) by a 23-g mouse. The mice were continued on treatment until they reached 21 weeks of age, and then they were euthanized by an overdose of sodium pentobarbital.

Measurement of Serum Cholesterol

Blood samples were collected from the heart, and after the blood was allowed to clot and serum was obtained, cholesterol was measured enzymatically by using a kit from Sigma Diagnostics (No. 352-20).

Tissue Preparation and Detection of Aortic Superoxide Anion

Entire thoracic and abdominal aortas were dissected in physiological buffer by using a dissecting microscope. The details of assay for aortic superoxide anion have been published previously.¹³ The aorta was incubated in test tubes containing 1 mL HEPES-buffered physiological solution (pH 7.4) with lucigenin (5 $\mu\text{mol}/\text{L}$) for measurement of superoxide anion. This low concentration of lucigenin was demonstrated not to be involved in redox cycling and to specifically indicate superoxide anion levels in vascular tissue.^{14,15} The luminometer was set to report arbitrary units of emitted light; after a 15-minute equilibration, repeated measurements were integrated every 30 seconds, and an average value was reported over a 5- to 10-minute period. Tiron (10 to 20 mmol/L), a cell-permeant nonenzymatic scavenger of superoxide anion, was then added to quench all superoxide anion-dependent chemiluminescence, and chemiluminescence was integrated over the last 2.5 minutes of an additional 5-minute period. Units of chemiluminescence were converted to picomoles of superoxide anion by standardization with xanthine oxidase and cytochrome c.¹⁶

Quantification of Aortic Atherosclerotic Lesion Area

Atherosclerotic lesions were quantified by planimetry of oil red O-stained lesions on the aortic intima. After measurement of aortic superoxide anion, the entire thoracic and abdominal aorta was rinsed with cold PBS, cut open longitudinally through its ventral side under a dissecting microscope, and immersed in oil red O (60% solubilized in propylene glycol). An additional incision was made at the aortic arch along the dorsal side from the aortic root to the left subclavian bifurcation, and the entire vessel was photographed. Quantification of stained lesion area was performed on the digitized images by using Scion Image software.

Statistical Analysis

Data are presented as mean \pm SE. Two-tailed Student *t* test for paired or unpaired samples assuming equal variances was used to compare treatments, and statistical significance was assumed at $P < 0.05$.

Results

S17834 Inhibits Induction of VCAM Surface Expression and U937 Adhesion by TNF

In HUVECs stimulated for 6 hours with TNF (0.1 to 100 U/mL), S17834 (10, 25, and 50 $\mu\text{mol}/\text{L}$) solubilized in DMSO had a concentration-dependent inhibitory effect on VCAM expression (Figure 1A). The maximum VCAM induction to 100 U/mL of TNF was inhibited by $49 \pm 9\%$ at 10 $\mu\text{mol}/\text{L}$, by $74 \pm 4\%$ at 25 $\mu\text{mol}/\text{L}$, and by 100% at 50 $\mu\text{mol}/\text{L}$. At the highest concentration used, there was no effect of the DMSO solvent on VCAM protein expression. A similar inhibitory effect was obtained when NaOH was used as the solvent (data not shown). At a concentration of 10 $\mu\text{mol}/\text{L}$, S17834 was similarly effective in reducing VCAM

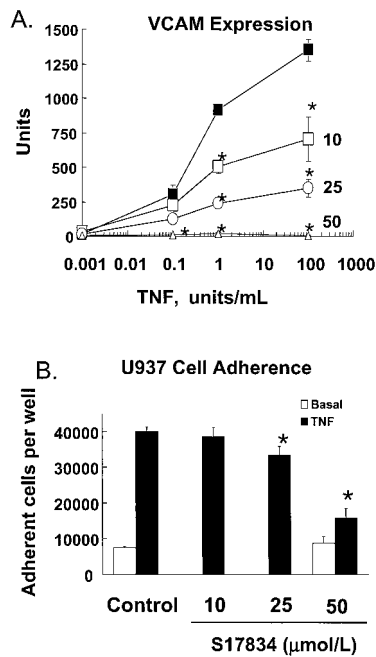


Figure 1. Concentration-dependent inhibitory effect of S17834 on VCAM cell surface protein expression and U937 cell adhesion. A, HUVECs were stimulated with TNF (0.1 to 100 U/mL) in the presence or absence of S17834 or its vehicle (0.1% DMSO). VCAM protein expression is expressed as arbitrary units of fluorescence. VCAM expression was significantly ($P < 0.05$) inhibited by S17834 at 10, 25, and 50 $\mu\text{mol/L}$. Values are mean \pm SEM from 3 experiments, each performed in triplicate. B, U937 cells labeled with Calcein were incubated with HUVECs that had been stimulated with TNF (1 U/mL) for 6 hours. The HUVECs were treated with solvent or S17834, which at 25 and 50 $\mu\text{mol/L}$, significantly decreased the adherence of U937 cells to TNF-stimulated HUVECs (solid bars). Basal adherence to HUVECs was unaffected by S17834 (50 $\mu\text{mol/L}$).

expression induced by TNF (100 U/mL) in human arterial endothelial cells ($40 \pm 5\%$ reduction).

We addressed the physiological relevance of the inhibitory effect of S17834 on VCAM expression by measuring the adherence of Calcein-labeled U937 cells to HUVECs stimulated with TNF (1 U/mL) for 6 hours. S17834 caused a concentration-dependent decrease in adhesion of U937 cells, with significant changes produced at 25 and 50 $\mu\text{mol/L}$ (Figure 1B). At 50 $\mu\text{mol/L}$, S17834 had no effect on basal adhesion of U937 cells (Figure 1B).

S17834 Inhibits VCAM, ICAM, and E-Selectin mRNA Expression Induced by TNF in HUVECs

To investigate whether regulation of CAM gene expression by TNF could be affected by S17834, mRNA accumulation for ICAM, VCAM, E-selectin, and PECAM was analyzed with Northern blots (Figure 2). Unlike VCAM and E-selectin, ICAM and PECAM mRNAs were expressed in resting cells. ICAM, VCAM, and E-selectin mRNA expression was increased when cells were incubated with TNF, whereas PECAM expression was not altered (Figure 2). Under these conditions, S17834 (50 $\mu\text{mol/L}$) significantly decreased ICAM, VCAM, and E-selectin expression. PECAM mRNA expression was unchanged by S17834 (Figure 2).

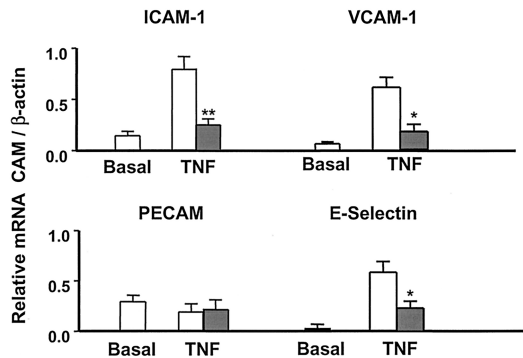


Figure 2. Northern blot analysis of ICAM, VCAM, PECAM, and E-selectin mRNA expression after incubation with TNF and S17834. HUVECs were stimulated by TNF (100 U/mL), in the presence of 50 $\mu\text{mol/L}$ S17834 (stippled bars) or its solvent (open bars), for 6 hours before extraction of total RNA. Basal expression in untreated cells was also assessed. mRNA for ICAM (3.4 kb), VCAM (3.2 kb), PECAM (2.6 kb), E-selectin (2 kb) and β -actin (2 kb) was quantified with an imaging densitometer. To correct for the difference in RNA loading and/or transfer, mRNA for adhesion molecules was normalized as a ratio to that of β -actin. In TNF-stimulated cells, S17834 significantly inhibited mRNA expression of ICAM, VCAM, and E-selectin, but not of PECAM. Data are from 4 different experiments (mean \pm SEM). $*P < 0.05$ and $**P < 0.01$ relative to solvent-treated cells stimulated with TNF.

S17834 Inhibits the Adhesion of Leukocytes on Recombinant CAM

The effect of S17834 on leukocyte adhesion was studied by using recombinant CAM-coated wells. After 90 minutes of incubation, S17834 (10 $\mu\text{mol/L}$) inhibited the adhesion of U937 monocytic cells to sVCAM and sP-selectin by $21 \pm 6\%$ ($n = 7$) and $33 \pm 6\%$ ($n = 5$), respectively. There was no significant effect of S17834 (10 $\mu\text{mol/L}$) on the adhesion of U937 to sE-selectin-coated wells ($6.3 \pm 4.8\%$, $n = 4$), but at 30 $\mu\text{mol/L}$, adherence was significantly reduced by $22 \pm 7.0\%$ (Figure 3). The adhesion of HSB₂ cells to sICAM-coated wells was inhibited by $51 \pm 5.3\%$ by S17834 (10 $\mu\text{mol/L}$). S17834 inhibited the adhesion of leukocytes to sICAM-, sP-selectin-, sVCAM-, and sE-selectin-coated wells, with

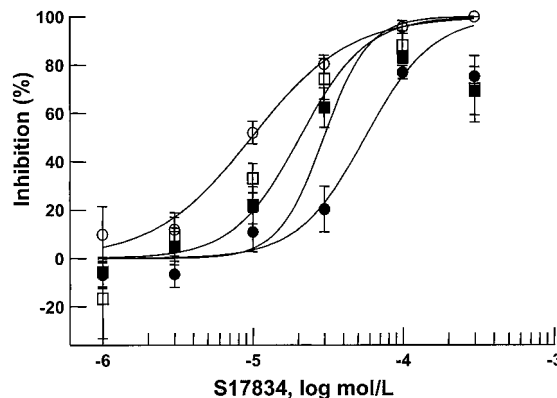


Figure 3. Concentration-dependent inhibitory effects of S17834 on the adhesion of U937 cells to sVCAM, sP-selectin, and sE-selectin and of HSB₂ cells to sICAM. S17834 (plotted as logarithm of the concentration) inhibited the adhesion of HSB₂ to sICAM-coated wells (open circles) and of U937 monocytic cells to sP-selectin-coated (open squares, $n = 5$), sVCAM-coated (solid squares, $n = 7$), and sE-selectin-coated (solid circles, $n = 4$) wells.

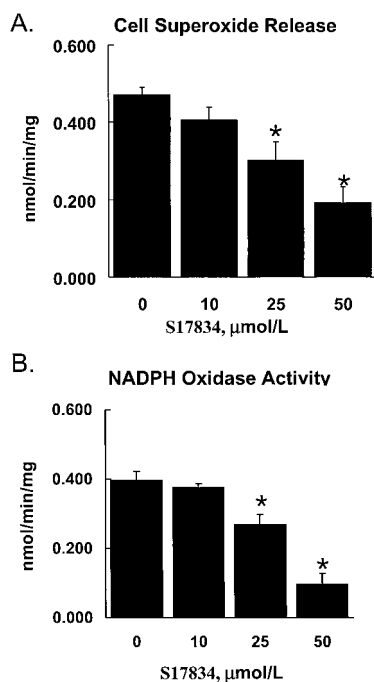


Figure 4. Effect of S17834 on cellular superoxide anion generation and membrane NADPH oxidase activity in HUVECs after stimulation with TNF. A, Superoxide anion production from intact HUVECs was determined by measuring the SOD-inhibitable reduction of acetylated horse heart type VI cytochrome *c* (80 μmol/L) to the ferrous form, which was measured at a wavelength of 550 nm. S17834 inhibited TNF (100 U/mL, 2 hours)–stimulated superoxide production at 25 μmol/L ($n=3$, $*P<0.03$) and at 50 μmol/L ($n=3$, $*P<0.005$). Values are mean \pm SEM. B, NADPH oxidase activity was determined in membranes obtained from cells stimulated with TNF (100 U/mL, 4 hours). S17834 at 25 and 50 μmol/L significantly reduced NADPH consumption by the membranes ($*P<0.02$, $n=3$).

IC₅₀ values of 9.8 \pm 1.3, 21 \pm 4.7, 30 \pm 4.1, and 56 \pm 9.6 μmol/L, respectively (Figure 3).

S17834 Inhibits Endogenous Endothelial Superoxide Anion Generation

To investigate whether S17834 inhibits TNF-stimulated CAM expression by an antioxidant mechanism, its effect on endogenous superoxide production was examined. TNF (100 U/mL) caused a significant increase in SOD-inhibitable cytochrome *c* reduction, indicating a stimulation of endogenous superoxide anion levels (0.24 \pm 0.02 [basal] and 0.47 \pm 0.03 [TNF] nmol \cdot min⁻¹ \cdot mg⁻¹, $P<0.04$). Similar to its effect on CAM expression and adhesion in TNF-stimulated cells, the augmentation in SOD-inhibitable cytochrome *c* reduction was prevented by S17834 in a concentration-dependent manner, with a significant reduction in superoxide anion levels at 25 μmol/L (0.32 \pm 0.04 nmol \cdot min⁻¹ \cdot mg⁻¹, $P<0.01$; $n=3$) and a return to basal levels at 50 μmol/L (0.19 \pm 0.04 nmol \cdot min⁻¹ \cdot mg⁻¹, Figure 4A).

To determine whether S17834 is a scavenger of superoxide anion, the effect of the drug was assessed on cytochrome *c* reduction caused by hypoxanthine (100 μmol/L) and xanthine oxidase. At 0.5 mU/mL xanthine oxidase, the rate of cytochrome *c* reduction was 7.8 \pm 0.4 mU/min, and at 1 mU/mL xanthine oxidase, the rate of reduction was 11.6 \pm 2.1 mU/min ($n=2$). In the presence of S17834 (50 μmol/L), these

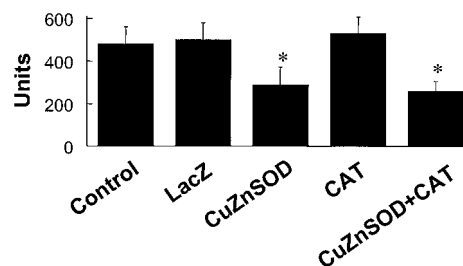


Figure 5. Effect of adenovirus-mediated overexpression of CuZnSOD and catalase (CAT) on TNF-induced VCAM expression. HUVECs were transfected with adenoviral vectors containing genes for LacZ, CuZnSOD, and CAT at a dose of 300 MOI for 48 hours preceding stimulation with TNF (100 U/mL) for 6 hours. Cell surface expression was not affected by Ad-LacZ but was significantly inhibited by Ad-CuZnSOD and inhibited to the same degree by transfection with Ad-CuZnSOD and Ad-Cat. No effect was observed with transfection of Ad-Cat alone. Values are mean \pm SEM expressed as arbitrary units of fluorescence from 4 (CAT and CuZnSOD+CAT) or 5 (CuZnSOD) experiments, each performed in triplicate. $*P<0.05$.

rates were unchanged (7.3 \pm 0.5 and 10.4 \pm 3.2 mU/min at 0.5 and 1 mU, respectively), indicating that the drug was neither a scavenger of superoxide anion nor an inhibitor of xanthine oxidase.

To assess the possibility that S17834 inhibits NADPH oxidase activity, its effect on the SOD-inhibitable reduction of cytochrome *c* by a membrane preparation of TNF-stimulated endothelial cells was measured. Figure 4B shows that S17834 caused a concentration-dependent decrease in NADPH oxidase activity, with significant decreases observed at 25 and 50 μmol/L. Diphenyleneiodonium (10 μmol/L), a nonspecific inhibitor of flavoproteins including NADPH oxidase, xanthine oxidase, and NO synthase, reduced activity \approx 80% to 0.093 \pm 0.041 nmol \cdot min⁻¹ \cdot mg⁻¹, a level similar to that reached with 50 μmol/L S17834. There was no effect of L-N^G-methyl arginine ethyl ester (1 mmol/L) on activity (0.41 \pm 0.03 nmol \cdot min⁻¹ \cdot mg⁻¹), indicating that there was no contribution of NO synthase to superoxide anion generation.

Effects of Superoxide Anion Scavenging by Tiron and Adenovirus-Mediated Overexpression of SOD and Catalase on VCAM Expression

To determine whether the effect of S17834 on VCAM expression is related to its ability to inhibit superoxide anion production, HUVECs were transfected with adenoviral vectors to increase the expression of CuZnSOD and catalase. Forty-eight hours after transfection, the activity of SOD was increased from 5 \pm 2 U/mg protein in LacZ-transfected cells to 30 \pm 2.3 U/mg protein ($n=3$) in cells transfected with 300 MOI of the CuZnSOD vector. Catalase activity was increased from 3.0 \pm 0.01 U/mg protein in LacZ-transfected cells to 1500 \pm 234 ($n=2$) in cells transfected with the catalase vector.

Transfection of cells with LacZ did not affect the expression of VCAM 6 hours after stimulation of TNF (100 U/mL, Figure 5). In HUVECs transfected with SOD, but not in those transfected with catalase, VCAM expression was significantly reduced (Figure 5). Transfection of HUVECs with catalase together with SOD did not affect the reduction in

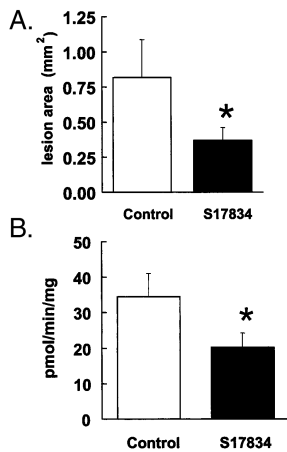


Figure 6. Effect of S17834 on aortic atherosclerotic lesion area and superoxide anion in apoE-deficient mice. A, Area of oil red O-stained lesions photographed and quantified on the intimal surface of the aorta by computerized image analysis. Treatment with S17834 ($130 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) significantly decreased lesion area. B, Superoxide generation by single mouse aortas measured under basal conditions with lucigenin chemiluminescence. S17834 treatment significantly decreased basal superoxide anion production by the aorta. Values are mean \pm SEM. * $P < 0.05$.

TNF-induced VCAM expression caused by transfection with SOD alone.

Similar to the effect of overexpression of SOD, Tiron (10 mmol/L), a superoxide anion scavenger, caused a significant reduction in VCAM expression caused by TNF (100 U/mL) to 230 ± 100 U versus 449 ± 158 U in HUVECs not treated with Tiron ($n=3$).

S17834 Inhibits Aortic Atherosclerotic Lesions and Superoxide Anion Generation in ApoE-Deficient Mice

Additional studies were performed to determine whether the inhibitory effect of S17834 seen on adhesion molecule expression and superoxide anion generation in endothelial cells could lead to a significant action of the drug on the development of atherosclerotic lesions in apoE-deficient mice. There was a significant ($>50\%$) reduction in total oil red O-stained lesion area in 21-week-old mice treated with $130 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ S17834 (area $0.37 \pm 0.09 \text{ mm}^2$, $n=14$) compared with that in untreated mice (area $0.82 \pm 0.27 \text{ mm}^2$, $n=12$; $P < 0.03$; Figure 6A). The results were similar when the data were analyzed as a ratio of the lesion area to total aortic area (0.013 ± 0.003 in treated mice compared with 0.029 ± 0.008 in untreated mice).

The antiatherogenic effect of S17834 was not due to an effect on serum cholesterol (409 ± 16 versus $465 \pm 27 \text{ mg/dL}$ in the treated and untreated mice, respectively) or nutritional status (for respective values in treated versus untreated mice, body weight 22.9 ± 0.3 versus $23.1 \pm 0.5 \text{ g}$ and heart weight 103 ± 1 versus $103 \pm 1 \text{ mg}$).

The antiatherogenic effect of S17834 was associated with a reduction in superoxide anion production by the aorta as detected by lucigenin. Under basal conditions, superoxide anion generation was $35 \pm 8 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ($n=16$) in

aortas of untreated mice compared with $20 \pm 1 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ in aortas of treated mice ($P < 0.05$; $n=23$, Figure 6B).

Discussion

S17834 demonstrated broad inhibitory effects on TNF-induced ICAM, VCAM, and E-selectin mRNA accumulation, VCAM protein cell surface expression, and the adherence of U937 monocytic cells. The inhibitory effect of S17834 on U937 and HSB₂ cell adherence to recombinant adhesion molecules also indicates that S17834 can target leukocyte adhesive mechanisms. The lack of effect of TNF and S17834 on PECAM expression indicates that S17834 selectively targets TNF-induced activation of endothelial cells.

Our results also indicate that the inhibitory effect of S17834 on the stimulation of human endothelial cells by TNF results, at least in part, from its ability to inhibit endogenous superoxide anion. This is suggested by the inhibition of TNF-induced VCAM expression in HUVECs with increased expression of CuZnSOD or in those treated with the superoxide anion scavenger, Tiron. The fact that the overexpression of catalase, either with SOD or alone, does not influence TNF-induced VCAM expression indicates that hydrogen peroxide, a product of SOD, is not involved.

It is apparent that in the concentrations used, S17834 is not a scavenger of superoxide anion. This was demonstrated by showing that S17834 did not affect superoxide production by xanthine oxidase in a cell-free system, a finding that also excludes an effect of the drug on this flavoprotein source of superoxide anion in TNF-stimulated HUVECs. The effect of S17834 on superoxide anion levels and VCAM surface expression is mimicked by diphenyleneiodonium, a nonspecific flavoprotein inhibitor.^{17,18} This effect of diphenyleneiodonium has been attributed to suppression of the activity of a neutrophil-like NAD(P)H oxidase, which is stimulated in endothelial cells by TNF.^{17,19,20} The inhibition of NADPH oxidase by S17834 was directly demonstrated in endothelial cell membranes, in which the potency of the drug was similar to that observed when its effect was tested on superoxide anion production in intact HUVECs stimulated with TNF. These studies show that S17834 inhibits specific endogenous sources of superoxide anion, including NADPH oxidase. Together with the studies using SOD overexpression, which directly implicate a regulatory role of superoxide anion in adhesion molecule expression, these studies suggest that the effect of S17834 on endothelial cell superoxide anion production is key to its antiadhesive effects.

To determine the effect of S17834 on leukocyte adhesive mechanisms at the endothelial and leukocyte interface, we tested its actions on U937 adherence to endothelial cell monolayers and to soluble CAM-coated plastic wells. The fact that the individual adhesion molecules that were affected by S17834 are involved in different stages of leukocyte recruitment (E-selectin and P-selectin), adherence (ICAM, VCAM, and E-selectin) and extravasation (VCAM and PECAM)²¹ indicates that S17834 has broad effects on endothelial cell-leukocyte adhesive mechanisms stimulated by TNF. Although other mechanisms that were not examined further in the present study may be involved, it is possible that the effects of S17834 are also exerted on leukocyte superoxide anion levels that increase on integrin binding.²²

Our observations are consistent with many reports indicating that the activation of endothelial cells by several different types of cell stimuli is regulated by redox-sensitive mechanisms. Cytokines (including TNF),²³ laminar shear stress or cyclic strain,²⁴ hypoxia/reoxygenation,²⁵ and exposure to elevated glucose^{26,27} and low density lipoproteins²⁸ have all been shown to increase the generation of superoxide anion in endothelial cells. How free radicals can regulate intracellular second messengers and modify cell function requires further investigation, but it is apparent from these studies that an agent that decreases superoxide generation has powerful effects on leukocyte adhesion. It is possible that the inhibition by S17834 of superoxide anion exerts its effect at the level of transcription factors such as nuclear factor- κ B or activator protein-1, whose activations are known to be sensitive to cellular redox status.²⁹

The expression of endothelial cell adhesion molecules, the influx of monocyte/macrophages, and the accumulation of lipids underlie the development of atherosclerosis, and they also indicate a process slowed by antioxidants.³⁰ The development of atherosclerotic lesions in apoE-deficient mice was dramatically decreased by S17834, demonstrating in vivo efficacy of long-term treatment with the drug. Although the mechanism by which S17834 exerted its effect in vivo is uncertain, the finding that superoxide anion levels were lower in the aorta suggests that mechanisms similar to those examined in vitro could be involved. Of course, such a change in superoxide anion may influence other processes involved in plaque development, such as fibroblast and smooth muscle proliferation, in addition to adhesion molecule expression. Indeed, NADPH oxidase, which is shown in the present study to be inhibited by S17834, has been implicated as having an essential role in a number of vascular disease states, including the hypertrophy and oxidant stress associated with angiotensin II-induced hypertension.³¹

In summary, the present study suggests that S17834 targets the generation of superoxide anion and, at least in part because of this, inhibits the early stages of TNF-induced activation of gene and protein expression of endothelial VCAM, ICAM, and E-selectin. The present and our previous studies^{10,32} indicating that S17834 inhibits ischemia/reperfusion injury suggest that S17834 could represent an important pharmacological tool that regulates leukocyte extravasation at the sites of inflammation in the macrovasculature and microvasculature. Potential clinical applications of such agents that reduce vascular inflammation might be quite broad, including atherosclerosis, ischemia/reperfusion-induced injury, and inflammatory venous disease.³³

Acknowledgments

This work was supported by National Institutes of Health grants HL-31607 and HL-55620 (Dr Cohen) and HL-51875 (Dr Cayatte) and by the Institut de Recherches Servier, Suresnes, France.

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Arterioscler Thromb Vasc Biol. 2001;21:1577-1584

doi: 10.1161/hq1001.096723

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:

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